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## **Autoantibody specificities and type I interferon pathway activation in Idiopathic Inflammatory Myopathies**

**Running head: Autoantibodies and type I Interferon in Myositis**

Louise Ekholm<sup>1\*</sup>, Saskia Vosslamber<sup>2\*</sup>, Anna Tjärnlund<sup>1</sup>, Tamarah de Jong<sup>2</sup>, Zoe Betteridge<sup>3</sup>, Neil McHugh<sup>3</sup>, Lenka Plestilova<sup>4</sup>, Martin Klein<sup>4</sup>, Leonid Padyukov<sup>1</sup>, Alexandre E. Voskuyl<sup>5</sup>, Irene E.M. Bultink<sup>5</sup>, D. Michiel Pegtel<sup>2</sup>, Clio P. Mavragani<sup>6,7</sup>, Mary K. Crow<sup>6</sup>, Jiri Vencovsky<sup>4</sup>, Ingrid E. Lundberg<sup>1</sup>, Cor L. Verweij<sup>2, 5†</sup>

\*Contributed equally

<sup>†</sup>deceased

<sup>1</sup>Rheumatology Unit, Department of Medicine, Karolinska University Hospital, Solna, Karolinska Institutet, Sweden

<sup>2</sup>Department of Pathology, VU University medical center, Amsterdam, the Netherlands

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<sup>3</sup>Pharmacy and Pharmacology, University of Bath, Bath, United Kingdom

<sup>4</sup>Institute of Rheumatology, Prague, Czech Republic

<sup>5</sup>Department of Rheumatology, Amsterdam Rheumatology and immunology Center, VU University medical center, Amsterdam, the Netherlands

<sup>6</sup>Mary Kirkland Center for Lupus Research, Hospital for Special Surgery, Weill Cornell Medical College, New York, NY, USA

<sup>7</sup>Department of Experimental Physiology, University of Athens, Athens, Greece

Corresponding author:

Louise Ekholm

Rheumatology Unit

Department of Medicine

Karolinska University Hospital,

17176 Stockholm, Sweden

E-mail: Louise.ekholm@karolinska.se

Ph: +46 517 74156

Fax: +46 8 517 73080

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## Abstract

Myositis is a heterogeneous group of autoimmune diseases, with different pathogenic mechanisms contributing to the different subsets of disease. The aim of this study was to test whether the autoantibody profile in myositis patients is associated with a type I interferon (IFN) signature, as in patients with systemic lupus erythematosus (SLE). Patients with myositis were prospectively enrolled in the study and compared to healthy controls and to patients with SLE. Autoantibody status was analyzed using an immunoassay system and immunoprecipitation. Type I IFN activity in whole blood was determined using direct gene expression analysis. Serum IFN inducing activity was tested using peripheral blood cells from healthy donors. Blocking experiments were performed by neutralizing anti-IFNAR or anti-IFN $\alpha$  antibodies. Patients were categorized into IFN high and IFN low based on an IFN score. Patients with autoantibodies against RNA-binding proteins had a higher IFN score compared to patients without these antibodies and the IFN score was related to autoantibody multi-specificity. Patients with dermatomyositis (DM) and inclusion body myositis (IBM) had a higher IFN score compared to the other subgroups. Serum type I IFN bioactivity was blocked by neutralizing anti-IFNAR or anti-IFN $\alpha$  antibodies. Concluding, a high IFN score was not only associated with DM, as previously reported, and IBM, but with autoantibody mono-specificity against several RNA-binding proteins and with autoantibody multispecificity. These studies identify IFN $\alpha$  in sera as a trigger for activation of the type I IFN pathway in peripheral blood, and support IFN $\alpha$  as a possible target for therapy in these patients.

## Introduction

Idiopathic inflammatory myopathies (IIM), also known as myositis, are rare chronic autoimmune diseases, characterized by proximal muscle weakness and muscle inflammation and can be subgrouped into polymyositis (PM), dermatomyositis (DM) and inclusion body myositis (IBM) [1, 2]. A

common feature is presence of autoantibodies, which is associated with distinct clinical phenotypes [3]. Some autoantibodies are exclusively found in myositis, so called myositis-specific autoantibodies, e.g., anti-Jo-1 and anti-SRP antibodies, whereas others, myositis-associated autoantibodies, such as anti-Ro52, anti-Ro60, anti-La, anti-PM/Scl, and anti-U1RNP antibodies, are also found in other autoimmune diseases [4]. Autoantibodies can present years before the onset of clinical symptoms indicating a role in the initiation of the disease but the mechanisms for this are as yet unknown [5].

The molecular mechanisms driving inflammation in patients with IIM are not fully understood. An activated type I interferon (IFN) pathway has been demonstrated in many autoimmune diseases [6] and has been proposed to be involved in the pathogenesis in IIM, particularly in the DM subgroup, but the mechanism driving the type I IFN pathway has not been clarified [7-12]. In general, plasmacytoid dendritic cells (pDCs) produce type I IFNs induced by viruses and also by immune complexes (ICs) consisting of autoantibodies directed against nucleic acids or nucleic acid binding proteins [13]. Autoantibodies against RNA-binding proteins have been associated with the type I IFN production in patients with systemic lupus erythematosus (SLE) [14-16]. We have previously demonstrated that sera from patients with PM and DM with anti-Jo-1 or anti-Ro52/60 autoantibodies, together with RNA, may act as endogenous IFN inducers in pDCs [17]. These observations suggest that there may be a role for the type I IFN system not only in DM, but also in other subtypes of myositis, where patients have antibodies against RNA or RNA-binding proteins and that autoantibodies may have a role in driving the type I IFN pathway in subsets of patients with IIM.

The aim of the present study was to test the hypothesis that autoantibodies directed against RNA-binding proteins in patients with myositis are associated with a type I IFN signature and thus potentially could induce IFN production. Moreover, we investigated the nature of the mediator that is responsible for the IFN activity in the blood of IIM patients.

## Patients and methods

This cohort consisted of prospectively enrolled patients between 2006 and 2009 from the Rheumatology Unit, Karolinska University Hospital, Stockholm, Sweden, and Institute of Rheumatology, Prague, Czech Republic, and fulfilled the criteria for definite or probable PM/DM [18, 19] or sporadic IBM [20]. Exclusion criteria were presence of overlap syndrome, treatment with biologicals and no available antinuclear antibody (ANA) status. A patient was defined as newly diagnosed when he or she had a maximum disease duration of a month and had no immunosuppressive treatment.

As a comparator group, 47 patients with SLE were recruited at the Rheumatology department, VU University medical center, Amsterdam, The Netherlands. Since the SLE cohort was used as a reference, patients with available SLE Disease Activity Index (SLEDAI) were included (SLEDAI 0, n=24, or SLEDAI >6, n=18). Forty-one healthy controls (HC), (23 (56%) female, mean age 35 years) were recruited at the VU University medical center, Amsterdam, The Netherlands.

This study was approved by the local ethics committees and informed consent was obtained from all subjects in the studies. Ethical permit Karolinska Hospital, Stockholm, Sweden: D-nr 2005/792-31/4 and 2011/1374-32, in VU Medisch Centrum, Amsterdam, the Netherlands: registration number 2007/125 and in Department of Rheumatology, Prague, Czech Republic: ref.nr 3233/2007.

### Clinical and laboratory data

The patients' overall disease activity at the time of serum sampling was assessed by the "Disease Activity Core Set Measures" according to the International Myositis Assessment and Clinical Studies (IMACS) Group [21, 22], based on prospectively collected data and retrieved from local myositis

databases at the Karolinska University Hospital and Institute of Rheumatology, Prague and from the web-based Euromyositis register, [www.euromyositis.eu](http://www.euromyositis.eu). Information on medication, malignancy (within 3 years before or after myositis diagnosis) and interstitial lung disease (ILD) was retrieved from the registries and from patient records. Interstitial lung disease (ILD) was defined as previously described [23]. Creatinine phosphokinase (CPK), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) levels in blood were analyzed as routine tests at the local Departments of Clinical Chemistry, and ANA was analyzed by immunofluorescence as a routine test at the Department of Clinical Immunology, Karolinska University Hospital, Sweden, or in the Laboratory Department of the Institute of Rheumatology, Prague.

#### **Autoantibody assays**

Sera in myositis patients were analyzed for autoantibodies against Jo-1, SRP, Mi-2, PM/Scl 70, PM/Scl 75, PM/Scl 100, PL7, EJ, Ku, Ro52, Ro60, La, and U1RNP using a validated line immunoassay system (Euroimmun, Lübeck, Germany), according to manufacturer's instruction. In addition, all patient sera were analyzed for autoantibodies against Jo-1, SRP, Mi-2, PM/Scl, Ku, PL7, PL12, EJ, OJ, Zo, KS, NXP2, TIF1 gamma, SAE, MDA5, RNAPI, RNAPII, RNAPIII, AMA, Topo, Ro60, La, U1RNP and U3RNP, using immunoprecipitation (IP) as previously described [24]. Lineblot is a reliable alternative to immunoprecipitation [25], but as there is some difference in specificities captured by the two methods, e.g. Ro52 we defined a patient as positive for an autoantibody if the autoantibody status was positive in one of the two used methods. Multi-specific antibody status was defined as presence of two or more autoantibodies (all specificities included), ANA not included. All tested antisynthetase autoantibodies (Jo-1, PL7, PL12, EJ, OJ, Zo, KS), in addition to antibodies against U1RNP and Ro60 were defined as autoantibodies against RNA-binding proteins.

### **IFN signature in whole blood**

For whole blood RNA isolation, 2.5 ml blood was drawn in PAXgene tubes (PreAnalytix, GmbH, Germany) and RNA isolation, quantification and purification were performed as previously described [26]. RNA (0.5 µg whole blood RNA derived from PAXgene tubes) was reverse transcribed into cDNA using a Revertaid H-minus cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturers' instructions. Real-Time PCR analysis was performed at ServiceXS (ServiceXS B.V., Leiden, The Netherlands) using the 96.96 BioMark™ Dynamic Array for Real-Time PCR (Fluidigm Corporation, San Francisco, CA, U.S.A), according to the manufacturer's instructions. Relative quantities were calculated using the ddCT method. GAPDH was used as a housekeeping gene and all arrays contained two samples for calibration.

Expression was determined of eight IFN-response genes (IRG), IFI3, IFIT2, MxA, IFI44L, HERC5, IFIT1, RSAD2 and OAS3, (all corrected versus GAPDH, log2 according to earlier studies [10, 12, 27]). Since the IRGs were highly correlative (Pearson  $r > 0.8$  for all combinations,  $p < 0.001$ ), we calculated an IFN score by averaging the expression levels of all IRGs per sample [28-30]. The mean + 2 standard deviations of the IFN score in HC was used as a cut off to define if an IFN signature was present (IFN high) or absent (IFN low).

### **IFN regulated gene (IRG) induction assay and neutralization**

Next we wanted to test if patient sera had the capacity to induce IFN activity. Peripheral blood mononuclear cell (PBMC) isolation from heparinized blood from one healthy donor (National blood bank, Netherlands) was performed using Lymphoprep (Axis Shield, Oslo, Norway) according to the



manufacturer's protocol [26]. After isolation, the PBMCs were cryopreserved in IMDM supplemented with 10% FCS and 10% DMSO and stored in liquid nitrogen until further use. Healthy donor PBMCs (with a cell concentration of  $2 \times 10^6$ /ml) were incubated with 20% sera from 20 randomly selected IIM patients for 4h or 8h at 37°C and 5% CO<sub>2</sub>. To identify the specificity of type I IFN in sera from a second subset of 25 randomly selected samples from IIM patients, 30 ng/ml neutralizing anti-IFN $\alpha$  antibody (#21105, PBL, Piscataway, New Jersey, USA) or 2.5  $\mu$ g/ml neutralizing anti-IFN $\alpha$ -receptor (IFNAR2) antibody (#21385, PBL, Piscataway, New Jersey, USA) were added to the cultures. After incubation, cells were harvested, washed and lysed in RLT buffer (Qiagen Benelux BV, Venlo, The Netherlands) according to the manufacturer's protocol. The lysates were stored at -20°C until RNA isolation. RNA isolation from cultured cells and reverse transcription of cDNA and Real Time qPCR was performed and assessed as previously described [26], with the exception that the expression levels of target genes were calculated relative to housekeeping gene 18S ribosomal RNA (18SrRNA). To correct for any variations between experiments, all expression values are relative to healthy controls. The overall IRG induction was determined by calculating the average expression of three known IRGs; RSAD2, IFI44L and MX1 [28, 29].

### **Genotyping**

Human leucocyte antigen (HLA) typing was performed for the myositis patients in order to investigate a possible link between HLA type and IFN activity. Sequence-specific primer-PCR (DR low-resolution kit; Olerup SSP, Saltsjöbaden, Sweden) was used for all Swedish patients. The PCR products were loaded onto 2% agarose gels for electrophoresis. An interpretation table was used to determine the specific genotype according to the recommendations of the manufacturer [31]. Allelic polymorphism of HLA-DRB1 and HLA-DQB1 genes for the Czech patients was analyzed by DNA-based typing using commercial sets (OneLambda, Los Angeles, USA) according to manufacturer's instructions.

## Statistical analyses

Data were analysed using GraphPad Prism 4 or 5 Software.

The significance of differences between groups was calculated by Mann-Whitney U test or student's t-test when appropriate for continuous variables, or by Pearson's Chi square tests or Fischer's exact test for categorical variables. Differences in IRG induction over time were tested using a paired t-test. Correlation analyses were done using Pearson r or Spearman r tests. P-values <0.05 were considered statistically significant.

## Results

### Patients

One hundred and eight patients with IIM were included, of which 29 were Czech and 79 were Swedish. Twelve of the Swedish patients were excluded according to the exclusion criteria and four patients based on missing IFN data due to technical issues. The remaining 92 were included. Ten of the Swedish patients and 2 of the Czech patients were newly diagnosed and were on no medications at the time of blood sampling. Patient characteristics at the time of blood sampling are shown in table 1. The most common antibody specificities were Jo-1 (n=23), Ro52 (n=22), Ro60 (n=10).

Antibody specificities in the clinical subdiagnoses of the 92 included patients, are presented in Supplementary Table 1.

### IFN activity in whole blood

The IFN activity was significantly higher in patients with myositis compared to HC ( $p=0.0007$ ), and equal to patients with SLE (Figure 1A). The patients were categorized into two groups, IFN high (n=41, 45%) and IFN low (n=51, 55%), based on the cut off value for the average gene expression in

HC. Patients with DM and IBM had higher IFN scores compared to PM patients ( $p=0.04$  and  $0.04$  respectively) (Figure 1B). There were large variations within these clinical subgroups.

### **IFN signature is related to autoantibody multi-specificity and to autoantibodies against RNA-binding proteins**

In order to search for a possible endogenous inducing factor of the type I IFN pathway we analyzed the relationship between the differential activation of the type I IFN pathway and presence of autoantibody specificities in IIM.

Twenty IIM patients had a multi-specific autoantibody status of whom 14 (70%) had an IFN signature, which was significantly more frequent than in patients with only one autoantibody specificity (18 out of 30 (60%),  $p=0.038$ ) and in patients with no detectable autoantibodies (27 out of 42 (63%),  $p=0.002$ ). In addition, a significantly higher IFN score was observed in the patients with multi-specific autoantibody status compared to those with only one ( $p=0.024$ ) or no ( $p=0.0098$ ) autoantibody specificities (figure 2A).

Autoantibody specificities in the patients with multi-specific autoantibody status are shown in supplementary table 2.

To determine whether the IFN signature was associated with distinct autoantibody specificities, patients with mono-specific autoantibody status were selected ( $n=30$ ). Autoantibody specificities and clinical subdiagnoses in patients with monospecific autoantibody status are shown in supplementary table 3. The IFN signature was clearly present in patients with mono-specificity for autoantibodies against RNA-binding proteins, such as anti-Jo-1 (55% positive for IFN signature), anti-Ro60 (50% positive) and anti-U1RNP (100% positive) autoantibodies, and was absent in most patients with mono-specificity for autoantibodies against other targets than RNA-binding proteins, such as, anti-Ro52 (20% positive for IFN signature) and anti-PM/Scl (20% positive). The IFN score was also

significantly higher for patients with autoantibodies against RNA-binding proteins compared to the other patients ( $p=0.011$ ) (Figure 2B), and the frequency of patients positive for an IFN signature was significantly higher in this group ( $p=0.003$ ).

### **IFN $\alpha$ in patients' sera is responsible for type I IFN pathway activation**

Next we investigated if sera from IIM patients (DM=9, PM=8, IBM =3, in total  $n=20$ ) had the capacity to activate the type I IFN pathway, i.e., upregulation of IRG expression in healthy donor PBMCs. The average ex-vivo whole blood IRG induction for all samples (1.96) was used to subdivide patients into IFN high and IFN low. Ten of the IIM patients' sera, defined as 'IRG high' showed type I IFN pathway-activating capacity four hours after serum-addition (mean 3.13) whereas significantly lower IRG induction was seen for the ten 'IRG low' patients (mean 1.41) (Figure 3A). The IFN high samples had a significantly higher expression of IRG compared to IFN low samples ( $p<0.0001$ ). Serum-induced IRG expression levels after four hours of incubation correlated positively with the IFN signature in whole blood ( $r=0.4$ ,  $p=0.005$ ). Similar observations were made with serum from SLE patients (data not shown). There were no significant differences in disease activity and autoantibody status between the IFN high and IFN low patients in these experiments. None of the patients' sera induced IRG expression eight hours after serum addition (Figure 3A).

In a second subset of IIM patients (DM=12, PM=7, IBM=6), in total  $n=25$ ) neutralizing anti-IFNAR or anti-IFN $\alpha$  antibodies were added to the sera and the type I IFN bioactivity was determined after four hours. In IRG high patient samples (DM=2, PM=1, IBM=2), but not in IRG low samples, IFN bioactivity was significantly inhibited by both anti-IFNAR antibodies ( $p=0.0160$ ) and anti-IFN $\alpha$  antibodies ( $p=0.0095$ ) confirming IFN $\alpha$  being mainly responsible for the type I IFN activity in these patients (Figure 3B). In both these experiments we did not see any significant statistical difference between the distribution of subdiagnoses of IIM in the IRG high and IRG low groups.

### **Correlation between IFN score and disease activity**

To explore whether an IFN signature could be associated with a clinical phenotype we investigated if the IFN score correlated to clinical manifestations. A low degree of correlation between the extent of the IFN score and disease activity (Physician's global disease activity assessment) was found for patients with DM (n=32,  $r=0.3778$ ,  $p=0.03$ ) (Figure 4) but not for the whole group with IIM, nor was any correlation seen between disease activity measures and IFN score for PM and IBM. We could not observe a correlation between the IFN score and other clinical or laboratory variables assessed (Table 2).

### **No association between HLA genotype and IFN signature**

We analyzed the association of presence of an IFN signature in patient serum with HLA-DRB1 genotype. No differences in frequencies of haplotypes of HLA-DRB1 were revealed between IFN high and IFN low patients.

### **Discussion**

The results in our study reveal an association between the type I IFN signature and a subgroup of myositis patients with autoantibodies against RNA-binding proteins, in addition to the previously reported association with the subdiagnosis DM. The association between the type I IFN signature and patients with autoantibodies against RNA-binding proteins was stronger in patients with more than one autoantibody specificity. We could demonstrate that IFN $\alpha$  activity is present in sera of subsets of IIM patients of all subdiagnoses and that IFN $\alpha$  can trigger the type I IFN pathway resulting in an IFN signature in peripheral blood of these patients.

The underlying pathogenesis of myositis has not yet been established. IFN $\alpha$  has been identified as a key cytokine predisposing to, and driving, SLE pathology [32] and prior studies suggest a similar phenomenon in myositis, particularly for the DM subgroup [7, 8, 33]. Several case reports on development of PM/DM during IFN $\alpha/\beta$  therapy have been published [34]. The cause and source of the IFN signature seen, particularly in myositis, have not been clarified [9, 10, 12]. Viral induction has been proposed, but causal relationship is lacking. Another explanation could be an endogenous trigger such as immune complexes as reported in patients with SLE or Sjögren's syndrome [16]. In juvenile DM (JDM) a correlation was shown between presence of autoantibodies against RNA-containing autoantigens and serum IFN $\alpha$  activity and the capacity to generate interferonogenic immune complexes (ICs) [35]. Similarly, we found that patients with autoantibodies against RNA-binding proteins had a higher IFN score in whole blood than patients without these autoantibodies. In addition, a higher IFN score was observed in the patients with multi-specific autoantibody status compared to those with only one or no autoantibody specificities, suggesting a dose response. The association with multispecificity could in 17 of 20 cases be explained by association to presence of autoantibodies against RNA-binding proteins. Additional support for a potential role of autoantibodies as inducing factors of the type I IFN system is the previously observed higher serum levels of IFN $\alpha$  in anti-Jo-1 autoantibody positive patients compared to anti-Jo-1 negative patients [11], as well as IFN $\alpha$ -inducing capacity of anti-Jo-1 serum samples [17]. These results are in line with reports from studies in SLE which have demonstrated that nucleic acid-containing ICs and their interaction with TLR7/TLR9 may induce the type I IFN pathway, suggesting that ICs containing RNA binding proteins may serve as endogenous inducers of type I IFNs. This could also be the case in patients with myositis [36, 37]. The association between autoantibody status and IFN score might suggest an IFN induction through ICs containing RNA and associated RNA binding proteins. However, some patient with DM and IBM did not have any detectable autoantibodies targeting RNA binding proteins, thus other mechanisms must also be involved. Other factors in sera, e.g., interleukins could also contribute to IFN activity as well as gene variants in the type I IFN signaling pathway [38].

We observed significantly more DM patients in the IFN high compared to the IFN low group, regardless of autoantibody status. Furthermore, patients with DM or IBM had a higher IFN score than PM, which is in line with previous observations [7, 8]. Upregulation of IFN-inducible genes has also been observed in the skin of patients with DM [39], and the presence of pDCs within the epidermis of DM skin suggests that the IFN-mediated processes may take place in the skin, as well as in the muscles of these patients by mechanisms other than the immune complex mediated mechanism discussed above [40].

By using blocking experiments we could demonstrate that IFN $\alpha$  present in the sera of IIM patients was responsible for the majority of the observed *in vitro* IRG induction, and that it is rapidly produced, within a few hours. Thus it is very likely that IFN $\alpha$  is present in the serum of IIM patients. A previous study has also shown a role for IFN $\beta$  in sera from DM patients [41]. However, in this study IFN $\beta$  was not measured.

In our study many patients did not display an IFN signature and were categorized as IFN low. One explanation could be that most of the patients were on treatment with glucocorticoids which are inhibitors of IFN production [42]. A study in JDM suggests that IFN $\alpha$  may be important in the early phase of disease [43]. Most patients in our study were, however, not in an early phase of their disease (mean disease duration of 3 years). It is also difficult to compare our IFN scores with previous studies, since different IFN assays have been used in different studies. However, we could show that the IFN score in myositis patients was equal to patients with SLE, a disease previously reported to be associated with a high IFN score [44]. A limitation of our study is the small sample size in the subgroup concerning autoantibody multispecificity, whereby it is difficult to draw complete conclusions.

There was only a weak correlation between the overall disease activity measure and the IFN score in blood for patients with DM, but not for the whole group with IIM, or for PM and IBM. There could be several explanations for this. One is that the overall disease activity was low due to ongoing immunosuppressive treatment. No association was observed between IFN score and age or sex (data not shown), therefore the HCs were not sex and age matched. Nor did we see an association between IFN score and other clinical or laboratory variables assessed (table 2).

No correlation was seen between HLA haplotype and presence of an IFN signature in this study, but this could be due to the limited number of patients. Similar results have been shown in JDM [43]. However, a large proportion of autoimmune disease risk genes are within the type I IFN signaling pathway [45].

In conclusion our study underscores that different molecular pathways may predominate in different subsets of myositis, emphasizing the need for careful molecular phenotyping of patients to gain better understanding of molecular pathogenesis and to improve treatment. The type I IFN pathway is activated in subsets of myositis patients with autoantibodies against RNA-binding proteins and in patients with DM/IBM, regardless of autoantibody status and in patients with autoantibody multispecificity. Thus the mechanisms driving the type I IFN pathway may differ between DM/IBM patients and patients with antibodies to RNA-binding proteins, where the latter group has a potential endogenous factor that can activate pDCs to produce type I IFN, whereas the mechanisms for IFN induction in patients with DM without these antibodies remain to be defined. IFN-blocking agents are on the market, and clinical trials with anti-IFN $\alpha$  monoclonal antibodies suggest beneficial effects in SLE [46], as well as in myositis [27]. The utility of a type I IFN gene signature (IFNGS) as a pharmacodynamic biomarker for assessing response to anti-IFN $\alpha$  monoclonal antibody treatment



has been suggested [27]. Preliminary results show a beneficial effect of target neutralization of the IFNGS and reduction in disease activity in DM and PM patients, but this needs additional confirmation in carefully phenotyped patients [27].

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## Tables

**Table 1.** Patient characteristics at time of blood sampling

Characteristics*	(n=92)
Diagnosis, n (%)	
PM	40 (43)
DM	46 (50)
IBM	6 (7)
Sex, n (%)	
Men/women	30 (33)/ 62 (67)
Age, years	58 (49-68)
Disease duration, years	3.0 (0.3-9.6)
Physician's global disease activity assessment, mm	7 (0-23)
Patient's global disease activity assessment, mm	36 (16-64)
MMT8, (0-100 %)	91 (72-99)
HAQ, (0.00-3.00)	0.50 (0.00-1.25)
CPK $\mu$ kat/liter	2.00 (1.05-8.23)
LDH $\mu$ kat/liter	3.60 (3.08-4.42)
Extramuscular global assessment, mm	5 (0-14)
ILD, n (%)	32 (35)
Malignancy, n (%)	9 (10)
Immunomodulatory drugs, n ( %)	55 (60)
Daily dose of Prednisolone, mg	10 (4-18)

\*All data are presented as median (IQR; interquartile range) if not stated otherwise.

PM: Polymyositis; DM: dermatomyositis; IBM: Inclusion Body Myositis; Disease duration: years from diagnosis till sampling date. Physicians global activity assessment (Visual Analogue Scale (VAS), from 0-100 mm); Patients global activity assessment (VAS from 0-100 mm); Extramuscular global assessment (VAS from 0-100 mm); MMT8: Manual muscle test (0-100 %); HAQ: Health Assessment Questionnaire (potential score 0.00-3.00); CPK: creatinine phosphokinase (normal levels: 0.6-3.5  $\mu$ kat/liter); LDH: lactate dehydrogenase (normal levels <3.5  $\mu$ kat/liter); ILD: Interstitial Lung Disease; Immunomodulatory drugs includes cyclophosphamide, methotrexate, azathioprine, cyclosporin A, and intravenous immunoglobulin; (n/a): not assessed.

**Table 2.** Characteristics of interferon (IFN) high and IFN low patients

Variables*	(n=92)		P value
	IFN high (n=41)	IFN low (n=51)	
Diagnosis, n (%)			
PM	13 (32)	27 (53)	DM vs other 0.2082
DM	24 (59)	22 (43)	
IBM	4 (9)	2 (4)	
Sex, n (%)			
Men/women	10 (24)/ 31 (76)	20 (39)/ 31 (61)	0.1991
Age, years	56 (49-71)	58 (49-68)	0.9896
Disease duration, years	3 (0.2-11.5)	4 (0.4-9)	0.7145
ANA positivity, n (%)	24 (59)	25 (49)	0.4845
Antibody profile†, n			
Jo-1	13	10	
PL7	1	0	

Ku	0	1	
Ro52	13	9	
Ro60	6	4	
PM/Scl 75/100	3	4	
SRP	1	1	
U1RNP	6	2	
La	3	1	
Physician's global disease activity assessment, mm	9 (5-22)	5 (0-28)	0.4426
Patient's global disease activity assessment, mm	29 (16-55)	37 (14-72)	0.4438
MMT8 (0-100 %)	90 (69-99)	86 (68-95)	0.2500
HAQ, (0.00-3.00)	0.44 (0-1.10)	0.75 (0.13-1.25)	0.1772
CPK, $\mu$ kat/liter	2.25 (1.23-8.90)	1.85 (1.13-7.16)	0.3214
LDH, $\mu$ kat/liter	3.90 (2.98-4.55)	3.5 (3.18-4.53)	0.4111
Extramuscular global assessment, mm	5 (0-13)	1.5 (0-11.5)	0.3855
ILD, n (%)	15 (37)	17 (33)	0.9161
Malignancy, n (%)	3 (7)	6 (12)	0.7262
Immunomodulatory drugs, n (%)	24 (59)	31 (61)	0.9963
Daily dose of prednisolone, mg	8 (15-19)	10 (3-19)	0.7177

\*All data are presented as median (IQR; interquartile range) if not stated otherwise.

IFN (interferon) high and IFN low; PM: Polymyositis; DM: dermatomyositis; IBM: Inclusion Body Myositis; Disease duration: years from diagnosis till sampling date; ANA: antinuclear antibody analysed by immunofluorescence; Physicians global disease activity assessment (Visual Analogue Scale (VAS) from 0-100 mm); Patients global activity disease assessment (VAS from 0-100 mm); Extramuscular global assessment (VAS from 0-100 mm); MMT8: Manual muscle test (0-100); HAQ: Health Assessment Questionnaire (potential score 0.00-3.00); CPK: creatinine phosphokinase (normal levels: 0,6-3,5 $\mu$ kat/liter); LDH: lactate dehydrogenase



(normal levels <3.5  $\mu$ kat/liter); ILD: Interstitial Lung Disease; Immunomodulatory drugs includes cyclophosphamide, methotrexate, azathioprine, cyclosporin A, intravenous immunoglobulin; (n/a): not assessed.

† Autoantibodies to Jo-1; PL7; Ku; Ro52; Ro60; PM/Scl 75/100; SRP; U1RNP; La. The other tested autoantibodies were negative. A patient is considered autoantibody positive if positive response in either the line immunoassay system or the immunoprecipitation assay. One patient could have several autoantibody specificities.

## Figure legends

**Figure 1 The interferon (IFN) score in idiopathic inflammatory myopathies (IIM) patients, systemic lupus erythematosus (SLE) patients and healthy controls (HC).** Gene expression levels of 8 IFN-response genes (IRG) were averaged to calculate the IFN score. The IFN score was measured in whole blood cells of IIM patients. The mean + 2\*standard deviations (SD) of the IFN score in HC (n=41) was used as a cut off to define if an IFN signature was present (IFN score > 4.84) or absent (IFN score < 4.84) (dotted line) **A)** The extent of the IFN score was compared between HC, SLE (n=47) and IIM patients (n=92) and an increased IFN score was observed in a subset of SLE and IIM patients. **B)** The extent of the IFN score was compared between IIM patients with different subdiagnosis, i.e. dermatomyositis (DM), polymyositis (PM) and inclusion body myositis (IBM). An IFN signature was observed in individual patients, irrespective of their subdiagnosis, but the IFN score was higher in DM (n=46) and IBM (n=6) patients compared to PM patients (n=40) (p= 0.0415 and 0.0415, respectively).

**Figure 2 Interferon (IFN) score in idiopathic inflammatory myopathies (IIM) patients with multi-specificity and RNA-binding autoantibodies.** Gene expression levels of 8 IFN-response genes were averaged to calculate the IFN score. The IFN score was measured in whole blood cells of IIM patients.

The mean + 2\*standard deviations (SD) of the IFN score in healthy controls (n=41) was used as a cut off to define if an IFN signature was present (IFN score > 4.84) or absent (IFN score < 4.84) (dotted line). **A)** The IFN score was compared between patients with multi-specific autoantibody profile (n=20) versus patients with mono-specific autoantibody profiles (n=30) and patients without autoantibodies (n=42). Almost all patients with multi-specific autoantibody profiles have an IFN signature and the IFN score was higher in those patients compared to patients with mono-specific autoantibody profiles (p=0.0240) and patients without autoantibodies (p=0.0098). **B)** Patients with autoantibodies against RNA-binding (n=17) proteins had significantly higher IFN-score compared to other patients (n=13) (p=0.011).

**Figure 3 Interferon (IFN) bioactivity in serum of idiopathic inflammatory myopathies (IIM) patients.**

- A)** To investigate the origin of the trigger or interferogenic component that is responsible for activation of the type I IFN pathway in sera of IIM, we tested sera of IIM patients for its capacity to activate the type I IFN pathway. In vitro upregulation of interferon gene regulated (IRG) expression was determined as a measure for the presence of type I IFN pathway-activating capacity in serum. Sera from IFN high patients induced IRG expression in peripheral blood mononuclear cells (PBMCs) from healthy donors after four hours of incubation, whereas sera from IFN low patients induced significantly lower IRG induction. None of the patients' sera induced IRG-expression at eight hours after serum-addition. "Baseline" indicates the basal IRG induction (0.27) in PBMCs that were not exposed to serum. The bars in the graph represent the mean of the three IRG genes, of all samples within the group, and the error bars represent the standard error of the mean. IRG high n=10, IRG low n=10.

**B)** To investigate whether type I IFNs are responsible for the observed activation of the type I IFN pathway in a subset of IIM patients (n=25), anti-IFN $\alpha$ -receptor (IFNAR) antibodies were added to the sera and the type I IFN bioactivity was determined after four hours. Serum-induced IRG expression, at four hours of incubation, was blocked when anti-IFN $\alpha$  or anti-IFNAR antibodies were added to the serum. A significant effect was observed for both anti-IFN $\alpha$  and anti-IFNAR antibodies in IRG high patients, but not in IRG low patients, indicating that IFN $\alpha$  in serum was responsible for the observed IRG induction. “Baseline” indicates the basal IRG induction (0.34) in PBMCs that were not exposed to serum. The bars in the graph represent the mean of the three IRG genes, of all samples within the group, and the error bars represent the standard error of the mean. IRG high n=5 and IRG low n=20.

**Figure 4 Correlation between Interferon (IFN) score and disease activity in patients with dermatomyositis (DM).** The IFN score was correlated to clinical manifestations. The mean + 2 standard deviation of the IFN score in healthy controls (HC) was used as a cut off to define if an IFN signature was present (IFN high) or absent (IFN low) and disease activity (Physician’s global disease activity assessment) measured as VAS (visual analogue scale) from 0-100mm, was observed for patients with dermatomyositis (DM) ( $r=0.3778$ ,  $p=0.03$ ,  $n=32$ ), but not for polymyositis (PM) and for inclusion body myositis (IBM), nor for the whole group of patients.



